

The Fractionation of Various Gelatin Preparations with Ion-Exchange Resins

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Gelatins and their fractions, separated by chromatography on carboxymethylcellulose after treatment with alkali, were analyzed by column chromatography using Dowex-50 and Dowex-1 ion-exchange resins.

An acid-processed pig skin gelatin was separated into about 30 distinct fractions by column chromatography on Dowex-50. Similar patterns were obtained with both biuret and ninhydrin reactions.

The ninhydrin reaction revealed some peaks which were not produced by the biuret reaction. The patterns of the three trypsin-hydrolyzed fractions from alkali-treated gelatin were all different.

It is concluded that the various heat-denatured products of collagens contain distinct fragments, which can be isolated with Dowex-1 and/or Dowex-50 resins for structural analysis.

The purposes of this work were (1) to obtain experience in the fractionation of collagen products with ion-exchange resins before and after treatment with trypsin and (2) to determine whether the three gelatin fractions obtained from alkali-treated gelatin by fractionation on carboxymethyl(CM-)cellulose¹ differed from each other.

EXPERIMENTAL

Material. The source of the collagen was the skin of a calf one week old. The soluble collagens were first removed by extraction with 0.45 *M* sodium chloride solution and subsequently with 0.15 *M* citrate buffer of pH 3.7. The residue was gelatinized in 0.1 *N* sodium hydroxide for 2 hrs at +90°C. This material was separated in CM-cellulose columns into fractions I, II and III.¹ The reference gelatin, from acid-processed pig skin, was a gift from Dr. J. E. Eastoe.²

Treatment with trypsin. Trypsin (Trypure, Novo) was added in the proportion of 1:100 (w/w) to a solution of gelatin in 0.1 *M* tris-HCl buffer of pH 8.0 and the mixture was incubated for 3 hrs at +37°C.

Fractionation with Dowex-50 resin.³ The resin, Dowex-50W × 2, 200–400 mesh from Fluka AG, Buchs SG, Switzerland, was fractionated before use by sedimentation⁴ and the particles with diameters in the range of 50–90 μ were selected. The resin was treated alternately with 1 *M* hydrochloric acid¹ and 0.2 *N* sodium hydroxide and finally washed with water.

The column (length 100 cm, diameter 1 cm) was kept at +37°C with a water-jacket. The resin in the sodium form was allowed to settle and about 200–300 ml of the eluant was allowed to run through. The flow of eluant was kept constant (8 ml/hr) with a peristaltic pump (C. Desaga GmbH, Heidelberg, Germany). Two-ml (15 min) fractions were collected with a fraction collector (Dr. Hans Hösli, Bischofszell, Switzerland) operated by a clock.

Twenty-milligram samples (pH 4.4) were allowed to run into the column. The run was carried out with the following solvents (heated in advance to +80°C and covered with liquid paraffin):

Tubes Nos. 1-96 (192 ml): The eluant was composed of 441 volumes of 0.1 *M* disodium phosphate and 559 volumes of 0.05 *M* citric acid containing 0.2 *M* of sodium chloride. The pH was 4.4.

Tubes Nos. 97-289 (384 ml): A gradient elution was arranged by adding gradually to the closed mixing vessel (volume 700 ml; fitted with a magnetic stirrer; contained originally the above-mentioned citrate-phosphate buffer) citrate-phosphate buffer of pH 7.03 composed of 829 volumes of 0.1 *M* disodium phosphate and of 171 volumes of 0.05 *M* citric acid containing 0.2 *M* sodium chloride. The final pH of the effluent was 5.8.

Tubes Nos. 290-385 (192 ml): The gradient elution was continued by introducing 0.2 *N* sodium hydroxide into the mixing vessel.

Tubes Nos. 386 onward: The eluant was 0.2 *N* sodium hydroxide.

Fractionation with Dowex-1 resin.^{5,6} The 60–110 μ fraction of Dowex 1 × 2, 200–400 mesh resin from Fluka AG was prepared, treated alternately with 1 *N* ammonia and glacial acetic acid, washed with deionized water, and allowed to settle in a column. The column was that used also for the Dowex-50 resin.

The twenty-milligram sample (pH 9.0) was applied and the column eluted as follows:

Tubes Nos. 1-68 (136 ml): The eluant contained 1 % collidine and 1 % pyridine brought to pH 9.05 with acetic acid;

Tubes Nos. 69-192 (248 ml): A gradient was applied. Acetic acid (0.1 *N*) was added to the closed mixing vessel (volume 250 ml) containing the above-mentioned pyridine-collidine buffer. The final eluant pH was 5.18.

Tubes Nos. 193-238 (376 ml): The gradient elution was continued, but now with 2.0 *N* acetic acid. The pH at the end of this stage was 3.0;

Tube No. 381 onward: The eluant was 2.0 *N* acetic acid.

Ninhydrin reaction. The method was a modification of Stein and Moore's procedure.⁷ The stannous chloride had to be omitted from the reagent, because stannous ion precipitated the phosphate present in the eluting buffers. The reagent contained instead 0.0002 % (w/v) of potassium cyanide.⁸ The colour intensity was measured at 570 m μ (5-cm light path).

Biuret reaction. A modified form of the original procedure of Lowry *et al.*⁹ was used. The alkaline copper-reagent was prepared by mixing 50 ml of a 6 % solution of sodium carbonate in 0.25 *N* sodium hydroxide and 1 ml of a 0.5 % solution of copper sulfate pentahydrate in 1 % sodium tartrate. Half a milliliter of the solution to be analyzed and 3.0 ml of the alkaline copper reagent were mixed and kept for 10 min. at room temperature. Diluted Folin's reagent (0.6 ml) was added and the colour measured at 750 m μ (5-cm light path) after a standing period of 30 min. at room temperature.

RESULTS

The reference gelatin, prepared from acid-processed pig skin and previously studied by Eastoe,² was fractionated first (Fig. 1). About thirty distinct fractions were obtained from this preparation which had not been treated with strong hydrolyzing agents or enzymes. When ninhydrin was used for the colour reaction, the fractions were resolved still better

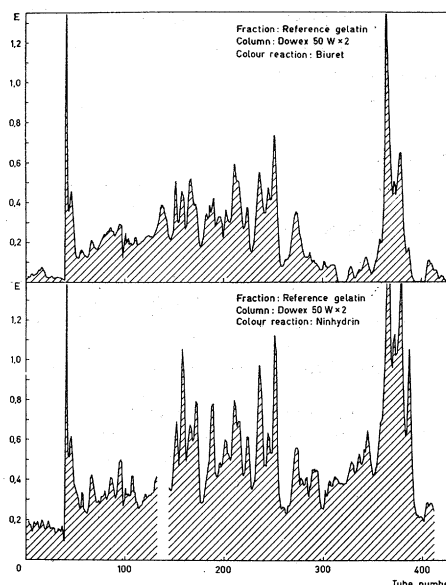


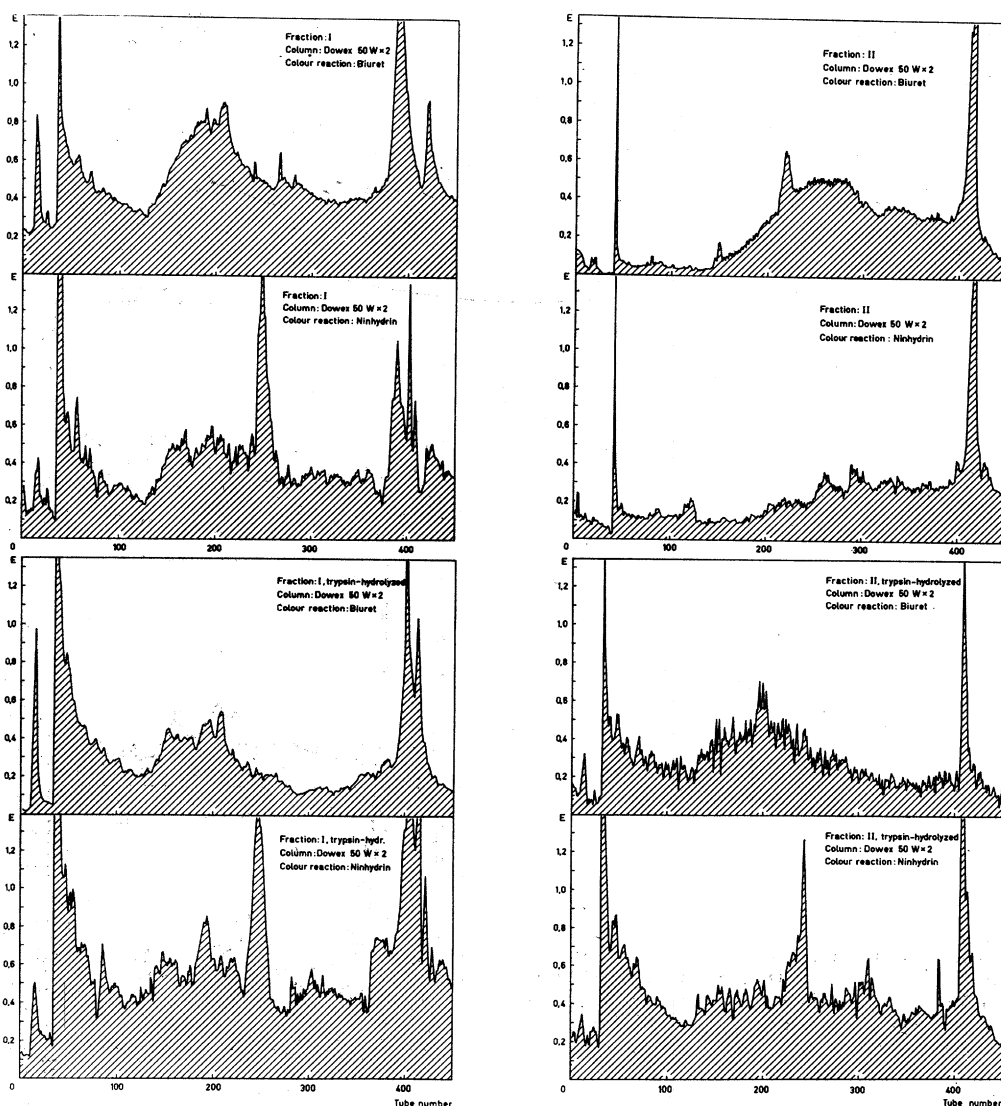
Fig. 1. Fractionation pattern of acid-processed pig skin gelatin eluted from a Dowex-50 column. For the eluants, see the experimental section.

but the pattern was essentially similar. It might be added that also the conventional »finger-print« on paper contained several distinct spots.

A complete collection of the patterns of the gelatin fractions I, II and III (obtained by CM-cellulose fractionation¹), both before and after tryptic hydrolysis, is shown in Fig. 2A-C. In Fig. 2A we observe separated fractions in all the patterns from fraction I, but that the resolutions is not as good as in Fig. 1. Both before and after tryptic hydrolysis there is a strong ninhydrin-positive peak (tubes Nos 230-250), perhaps containing a small peptide. More distinct peaks were produced with ninhydrin, but the differences were not marked. The effect of the treatment with trypsin is surprisingly slight, but most apparent in the tubes Nos. 350-410, as expected.

In regard to fraction II (Fig. 2B) similar observations were made. The ninhydrin reaction yields more highly resolved peaks. The effect of trypsin is small, except that in the ninhydrin-produced pattern there appears a strong peak at the tubes Nos. 220-250.

Fig. 2C shows that the bulk of the collagen remains in the fraction III. The ninhydrin reaction gives a peak (tubes Nos 25-40) which is not present in the pattern obtained with



the biuret reaction. Both patterns show after hydrolysis with trypsin about twenty more or less distinct fractions. A new large ninhydrin-positive peak appears at tubes Nos. 300-330.

When the patterns for fractions I, II and III are compared, it is seen that fraction I contains relatively much material that passes through the column rapidly (tubes Nos 1-140). The patterns of fractions II and III resemble each other. The pattern produced by the ninhydrin reaction after the treatment with trypsin, represents a »finger-print» of the peptides

DISCUSSION

In the literature there are numerous papers on the fractionation of tryptic hydrolysates of collagen and of other proteins as a step toward complete structural analysis.^{5,6,10,11} The novel point of the present experiments is the fractionation of heat-denatured collagens to distinct fragments without any enzymic treatment. It may be mentioned that

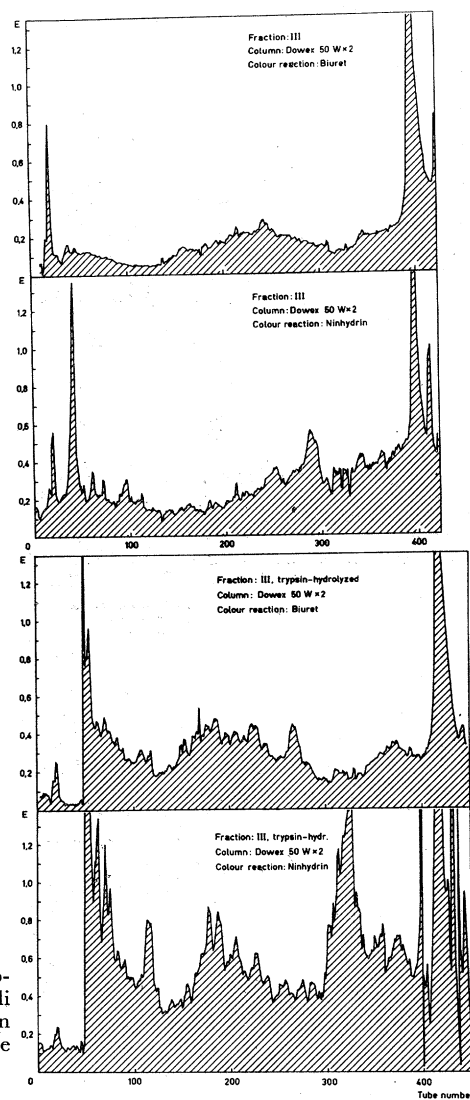


Fig. 2. A - C. Fractionation patterns (Dowex-50 column) of fractions I, II and III obtained from alkali treated calf skin collagen by preliminary fractionation on carboxymethylcellulose columns. For the eluants, see the experimental section.

of these subfractions. The original hypothesis that the fractions I, II and III contain fragments from different parts of the molecules seems tenable because the patterns are all different (tubes Nos: 80-120, 170-180, 230-250, 300-320 considered together).

The fractionation pattern obtained with the basic ion-exchange resin Dowex-1 are different from those obtained with Dowex-50 (Fig. 3A *vs.* Fig. 2A; Fig. 3B *vs.* Fig. 2C). The effect of trypsin is quite marked. Fractions I and III did not differ much. only remnants of the original α and β subunits were identified by electrophoretic methods among the breakdown products of collagen or commercial gelatins.¹² After prolonged denaturation at $+40^{\circ}\text{C}$ new electrophoretic fractions are broken from collagen.¹³

For the structural analysis of collagen these procedures offer the further possibility of obtaining distinct fragments by heating collagen at $+90^{\circ}\text{C}$ for 1—2 hrs in mild acidic or alkaline conditions, each of which can be purified further by chromatography either on other ion-exchange resins or on ion-exchange celluloses and finally degraded with suitable enzymes.

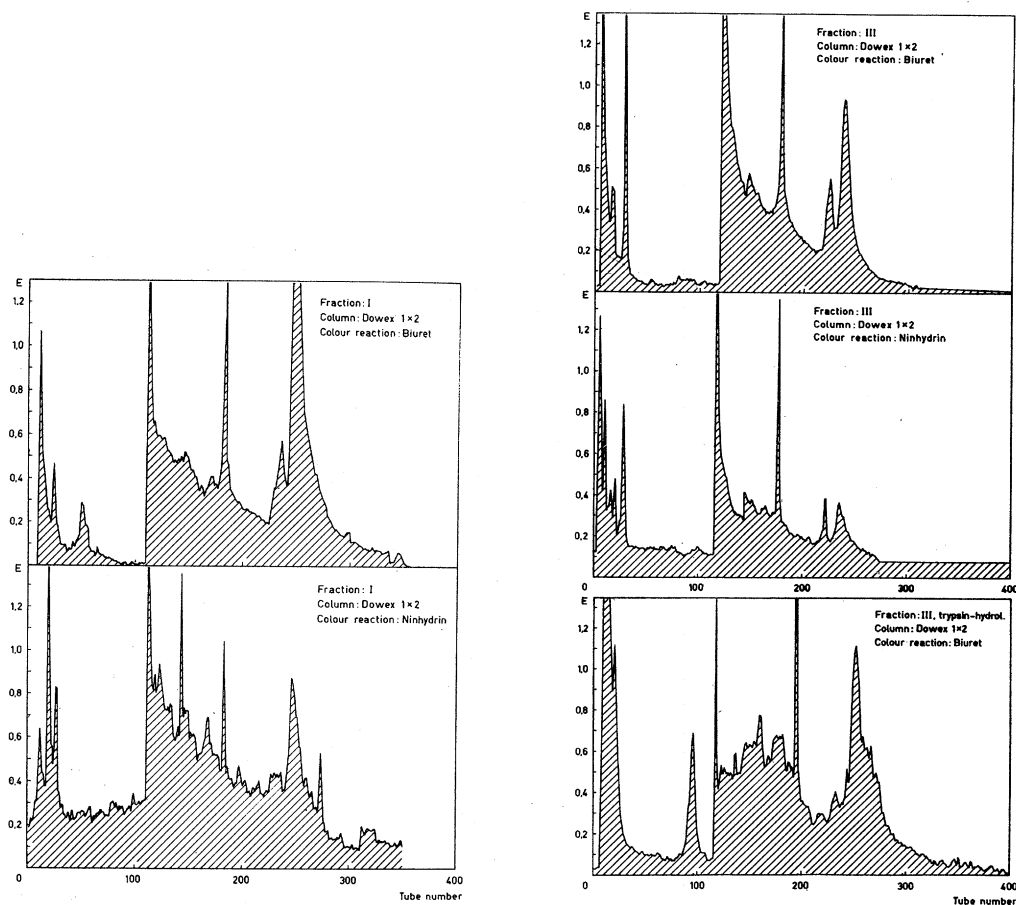


Fig. 3. A - B. Fractionation patterns (Dowex-I column) of the fractions I and III obtained from alkali-treated calf skin collagen by preliminary fractionation on carboxymethylcellulose columns. For the eluants, see the experimental section.

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